Kinetic characterization of recombinant PfAM1, a M1-aminopeptidase from *Plasmodium falciparum* (Aconoidasida: Plasmoziidae), using fluorogenic peptide substrates

Caracterización cinética de la PfAM1 recombinante, una aminopeptidasa M1 de Plasmodium falciparum (Aconoidasida: Plasmoziidae), con sustratos peptídicos fluorogénicos

Jorge O. González-Bacerio, Adriana K. Carmona, Marcos L. Gazarini, María Á. Chávez and Maday Alonso del Rivero

*Centro de Estudio de Proteínas, Facultad de Biología, Universidad de La Habana, La Habana, Cuba. Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, Brasil. Departamento de Biociências, Universidade Federal de São Paulo, Santos, São Paulo, Brasil.

* Autor para correspondencia: maday@fbio.uh.cu

**ABSTRACT**

*Plasmodium falciparum* neutral M1-aminopeptidase (PfAM1) is a validated target against malaria, the main human parasitic disease in tropical regions. Recently, our group obtained a recombinant variant of this enzyme (rPfAM1) in *Escherichia coli* with a yield of 24 mg of active protein per L of culture. As a way to confirm the suitability of this recombinant aminopeptidase to be used as a target for the identification of new potent and selective inhibitors of the endogenous enzyme, and adjusting the experimental parameters of the inhibition assays, here we determined the main kinetic characteristics of rPfAM1 using L-aminoacyl-7-amido-4-methylcoumarin (AMC)-conjugated fluorogenic substrates. Firstly, the Zn$^{2+}$ cation inhibited rPfAM1 activity at concentrations > 1 μmol/L, an effect qualitatively analogous to that observed for other metallo-aminopeptidases. In agreement with earlier reports, the near-neutral pH (7.2-7.4) is the optimum against the 4 substrates tested (Ala-, Leu-, Met- and Arg-AMC). The K$_M$ values at pH 7.2 (Met-AMC: 23 ± 5 μmol/L; Arg-AMC: 37 ± 4 μmol/L; Ala-AMC: 106 ± 9 μmol/L; Leu-AMC: 60 ± 7 μmol/L) are closer to those previously reported for the native enzyme than those described for another recombinant variant of PfAM1. On the other hand, the k$_{cat}$ values obtained here at pH 7.2 (Met-AMC: 99 ± 9 s$^{-1}$; Arg-AMC: 129 ± 6 s$^{-1}$; Ala-AMC: 268 ± 20 s$^{-1}$; Leu-AMC: 79 ± 6 s$^{-1}$) are the highest reported to date for PfAM1, indicating that our enzyme preparation is particularly active. Diminution of pH from 7.2 to 5.2, affected more the catalytic efficiency of rPfAM1 than affinity toward Leu-, Met- and Arg-AMC. The contrary effect was observed with Ala-AMC. The kinetic parameters at near-neutral pH varied as a function of the identity of the substrate amino-terminal residue, in a
similar way to that reported by another group using natural dipeptide substrates against a third reported recombinant form of PfAM1. Finally, the rPfAM1 here obtained was not sensitive to the inhibitors PMSF, E64 and pepstatin A, but was inhibited by amastatin, 1,10-phenanthroline and bestatin. This is an inhibition profile typical of neutral and basic metallo-aminopeptidases, also reported for native PfAM1. The obtainment of the kinetic parameters of rPfAM1 toward aminoacyl-AMC peptides will allow using these substrates to assess the inhibition of the endogenous enzyme on isolated live parasites.

Keywords: malaria, kinetic characterization, kinetic parameters, M1-family aminopeptidase, fluorogenic peptide substrates, AMC, Plasmodium falciparum, PfAM1

RESUMEN
La aminopeptidasa M1 neutra de Plasmodium falciparum (PfAM1) es un blanco validado contra la malaria, la principal enfermedad parasitaria humana en las regiones tropicales. Recientemente, nuestro grupo obtuvo una variante recombinante de esta enzima (PfAM1r) con un rendimiento de 24 mg de proteína activa por L de cultivo. Para confirmar la conveniencia de esta aminopeptidasa recombinante como blanco para la identificación de nuevos inhibidores potentes y selectivos de la enzima endógena, y para ajustar los parámetros experimentales de los ensayos de inhibición, en este trabajo se determinaron las principales características cinéticas de la PfAM1r frente a sustratos fluorogénicos conjugados L-aminoacil-7-amida-4-metilcumarina (AMC). En primer lugar, el catión Zn$^{2+}$ inhibió la actividad de la PfAM1r a concentraciones > 1 μmol/L, un efecto análogo cualitativamente al observado para otras aminopeptidásas de tipo metalo. En correspondencia con informes anteriores, el pH cercano al neutro (7,2-7,4) es el óptimo con los 4 sustratos evaluados (Ala-, Leu-, Met- y Arg-AMC). Los valores de K_m al pH 7,2 (Met-AMC: 23 ± 5 μmol/L; Arg-AMC: 37 ± 4 μmol/L; Ala-AMC: 106 ± 9 μmol/L; Leu-AMC: 60 ± 7 μmol/L) son más cercanos a los informados previamente para la enzima nativa que los obtenidos por otro grupo de autores con otra PfAM1 recombinante. Por otra parte, los valores de k_cat obtenidos en este trabajo a pH 7,2 (Met-AMC: 99 ± 9 s⁻¹; Arg-AMC: 129 ± 6 s⁻¹; Ala-AMC: 268 ± 20 s⁻¹; Leu-AMC: 79 ± 6 s⁻¹) son los mayores informados hasta ahora para la PfAM1, lo que indica que la preparación enzimática obtenida es particularmente activa. La disminución del pH de 7,2 a 5,2, afectó más la eficiencia catalítica de la PfAM1r que la afinidad frente a la Leu-, Met- y Arg-AMC. El efecto contrario se observó con la Ala-AMC. Los parámetros cinéticos a valores de pH cercanos al neutro variaron en función de la identidad del residuo amino terminal del sustrato, de manera similar a lo informado por otro grupo, los que utilizaron una tercera PfAM1 recombinante y sustratos dipéptidos naturales. Finalmente, la PfAM1r no resultó sensible a los inhibidores PMSF, E64 y pepstatina A, pero se inhibió por la amastatina, la 1,10-fenantroline y la bestatina. Este es un perfil de inhibición típico de aminopeptidásas de tipo metalo neutras y básicas, informado también para la PfAM1 NATIVA. La obtención de los parámetros cinéticos de la PfAM1r frente a los péptidos aminoacyl-AMC permitirá el empleo de estos sustratos para evaluar la inhibición de la enzima endógena en parásitos vivos aislados.

Palabras clave: malaria, caracterización cinética, parámetros cinéticos, aminopeptidasa de la familia M1, sustratos peptídicos fluorogénicos, AMC, Plasmodium falciparum, PfAM1

INTRODUCTION
Metalloproteases constitute the most diverse catalytic class of proteases, are broadly spread across living beings, and contain in their active site at least one divalent metallic cation (generally Zn$^{2+}$, Co$^{2+}$ or Mn$^{2+}$) acting as a ligand for catalytic nucleophilic water (Barret et al., 2003). Among these enzymes, metallo-aminopeptidases (APs) hydrolyze peptide bonds from the amino termini of polypeptide chains. Neutral metallo-APs are a distinctive group which develops crucial biological functions for different organisms, including their essential involvement in the life cycles of different parasites. Therefore, neutral metallo-APs are recognized as targets in relevant human parasitic diseases. For example, a leucyl AP belonging to the M17 family of metalloproteases was identified as a target in the kinetoplastid parasite Trypanosoma
cruzi, the causative agent of Chagas disease (Cadavid-Restrepo et al., 2011). The ortholog of this enzyme is also present in the genus Leishmania, which causes leishmaniasis (Morty and Morehead, 2002). Similarly, the protozoan unicellular parasite Plasmodium falciparum contains two neutral metallo-APs, belonging to the M1 (PfAM1) and M17 families, which have emerged as promising targets in malaria (Florent et al., 1998; Gardiner et al., 2006; Harbut et al., 2011).

Native PfAM1 (nPfAM1) (EC 3.4.11.2) is a monomeric enzyme of 1085 amino acids and 126 kDa (primary protein product) (Florent et al., 1998; Allary et al., 2002), structured in 4 domains (McGowan et al., 2009). It has a general fold very similar to that of bacterial neutral metallo-APs (Addlagatta et al., 2006). PfAM1 is expressed in the erythrocytic stages of the parasite life cycle as two active and soluble processed forms of 96 and 68 kDa (Florent et al., 1998; Allary et al., 2002; Azimzadeh et al., 2010), and has been localized on the cytosol (Allary et al., 2002; McGowan et al., 2009), digestive vacuole and nucleus (Dalal and Klemba, 2007; Ragheb et al., 2011) of P. falciparum. This AP contains a single Zn²⁺ ion in the active site (McGowan et al., 2009), has a pH optimum of 7.0-7.4 (Allary et al., 2002; McGowan et al., 2009), and it is specific for peptides (natural or artificial) with basic and hydrophobic residues at the amino terminus (P1 position) (Allary et al., 2002; McGowan et al., 2009; Dalal et al., 2012; Porbea et al., 2012; González-Bacerio et al., 2014).

This essential and non-redundant enzyme (Dalal and Klemba, 2007) participates in vacuolar (Ragheb et al., 2011) and/or cytosolic (Kolakovich et al., 1997) cleavage of dipeptides derived from the degradation of the host hemoglobin. This process is nutritionally necessary for parasite development during their clinically-relevant asexual erythrocytic stages (ring, trophozoite, schizont and merozoite) (Liu et al., 2006), and has been profusely targeted for antimalarials development (Yeh and Altman, 2006). In addition, it has been proposed that PfAM1 plays a role on ring-trophozoite transition (Harbut et al., 2011), trophozoite development (Gavigan et al., 2001), erythrocyte reinvasion (Allary et al., 2002), and has an unknown function in the nucleus (Dalal and Klemba, 2007; Ragheb et al., 2011).

Since the purification of nPfAM1, to obtain enough amounts for kinetic studies and further searching for inhibitors, is a difficult task, the enzyme has been expressed by several groups using the heterologous system Escherichia coli (McGowan et al., 2009; Azimzadeh et al., 2010; Ragheb et al., 2011). All these recombinant proteins are truncated forms of PFAM1, and only the variants produced in 2009 and 2011 (termed here rPFAM1₁⁹²⁻¹₀₈₅ and rPFAM1₁⁹²⁻¹₀₈₅, respectively) were kinetically characterized using fluorogenic substrates (McGowan et al., 2009; Ragheb et al., 2011; Porbea et al., 2012). Since their kinetic properties are similar to those of nPFAM1 (Allary et al., 2002), these proteins have been used as targets for the identification of inhibitors (McGowan et al., 2009; Harbut et al., 2011; Velmourougane et al., 2011; Skinner-Adams et al., 2012; Kannan Sivaraman et al., 2013).

By using a synthetic gene optimized for the E. coli system, recently we expressed high levels of another recombinant variant of PFAM1 (rPFAM1) in the cytosol of E. coli BL21 (González-Bacerio et al., 2014). This protein has a predicted molecular mass of 106.13 kDa and two six-histidine tags (at the N- and C-termini), allowing its purification in a single step by immobilized metal ion affinity chromatography (IMAC). Here we report the kinetic characterization of rPFAM1 toward four L-aminoacyl-7-amido-4-methylcoumarin (AMC) fluorogenic peptide substrates. This study allows (i) confirming the possibility of using rPFAM1 as a model of the endogenous enzyme for the identification of new PFAM1 inhibitors, (ii) to establish the appropriate experimental conditions for the inhibition assays, and (iii) the further use of these substrates to test the inhibition of nPFAM1 on isolated live parasites.

MATERIALS AND METHODS

Obtainment of rPFAM1

rPFAM1 was expressed in E. coli and purified in a single IMAC step as was previously described (González-Bacerio et al., 2014).

Protein concentration assay

Concentration of rPFAM1 was determined by the Bradford method, using bovine serum albumin as standard protein, and corroborated from absorbance at 280 nm using the extinction coefficient of rPFAM1₁⁹²⁻¹₀₈₅ previously reported: 1.15 x 10⁻³ L·mol⁻¹·cm⁻¹ (Ragheb et al., 2011).

Aminopeptidase activity assay

Aminopeptidase enzymatic activity (EA) was determined by a continuous kinetic method, using Ala-, Leu-,
Arg- or Met-AMC fluorogenic substrates (Amino Tech P & D, Brazil) solubilized in dimethyl sulfoxide (DMSO). The fluorescence due to the release of AMC (excitation: 380 nm, emission: 460 nm, slits: 10 nm) was monitored during 10 min using a microplate spectrophotometer (Hitachi F7000, Japan). Kinetic assays were carried out with concentrations of rPfAM1 that were linearly related to the initial rates, at 37°C, in 96-well black plates (200 μL final volume). The activity buffers were: 50 mmol/L Tris-HCl pH 7.2, for the assays performed at this pH; and constant ionic strength universal buffer (25 mmol/L acetic acid, MES and glycine, 75 mmol/L Tris), for pH optimum determination and the assays performed at pH 5.2. The final concentration of DMSO was less than 2% (v/v). Only the linear portions of progress curves, corresponding to a substrate consumption lower than 5%, were used to measure the reaction rates. Slopes with R² < 0.98 were not considered. The proportionality constant between fluorescence and AMC concentration was determined by fully converting 1, 2, 5 and 10 μmol/L Leu-AMC to products, measuring the final fluorescence and subtracting the background values corresponding to the non-hydrolyzed substrate controls. All assays were performed by triplicate.

Assessment of effect of Zn²⁺ concentration on rPfAM1 enzymatic activity

The effect of the Zn²⁺ concentration on the EA of rPfAM1 was evaluated in a range of 0.01-100 μmol/L ZnCl₂ (Sigma, USA), at pH 7.2 using 1.7 x 10⁻¹⁰ mol/L rPfAM1 and 34.7 μmol/L Leu-AMC. The remaining experimental conditions were equal to those above described. Residual activity was defined as the ratio between the reaction rates in the presence and in the absence of exogenous Zn²⁺.

Determination of pH optimum for rPfAM1

The effect of pH was evaluated from pH 4.0 to 11.0, using universal buffer, 1.7 x 10⁻¹⁰ mol/L rPfAM1, and 406.3 μmol/L Ala-AMC (~2 Kₐ for nPfAM1; Allary et al., 2002), 347.1 μmol/L Leu-AMC (~6 Kₐ for nPfAM1), 302 μmol/L Arg-AMC (~3 Kₐ for nPfAM1) or 326.7 μmol/L Met-AMC (Kₐ not determined for nPfAM1). The experimental conditions were equal to those above described. Relative activity was defined as the ratio between the reaction rate at a given pH and the maximal rate measured.

Determination of kinetic parameters of rPfAM1

The kinetic parameters of rPfAM1 were determined at pH 7.2 and 5.2, in order to in vitro reproduce the electrostatic microenvironment of the active site of nPfAM1 in the cytosol and the food vacuole (Kuhn et al., 2007) of P. falciparum, respectively. Substrate selection was based on the known specificity of this enzyme (Allary et al., 2002; McGowan et al., 2009; Dalal et al., 2012; Poreba et al., 2012; González-Bacerio et al., 2014).

Assays were performed with 1.7 x 10⁻¹⁰ mol/L rPfAM1 (at pH 7.2) and 4.2 x 10⁻¹⁰ mol/L rPfAM1 (at pH 5.2), at 8 concentrations of each substrate prepared by serial dilutions and spanning the following ranges: Ala-AMC (3.2-406.3 μmol/L), Leu-AMC (2.7-347.1 μmol/L), Arg-AMC (2.4-302 μmol/L), Met-AMC (2.6-326.7 μmol/L). The experimental conditions were equal to those above described. The kinetic parameters Michaelis-Menten’s constant (Kₐ) and catalytic constant (kcat) were calculated by fitting the experimental data to the Michaelis-Menten’s rectangular hyperbola function (Copeland, 2000) using GraFit software (version 5.0.13; Erithacus Software Limited [http://www.erithacus.com/grafit]). Results are presented as means ± SE.

Determination of rPfAM1 inhibition profile

The inhibition profile of the rPfAM1 activity was determined using the inhibitors (Sigma, USA) PMSF (2 mmol/L), E64 (5 μmol/L), pepstatin A (50 μmol/L), which recognize serine-, cysteine- and aspartic proteases, respectively, in addition to amastatin (10 μmol/L, metallo-APs inhibitor), 1,10-phenanthroline (1 mmol/L, metallo-proteases inhibitor) and bestatin (20 μmol/L, inhibitor of neutral and basic metallo-APs; Bachem, Sweden). The enzyme-inhibitor mixtures, containing 1.7 x 10⁻¹⁰ mol/L rPfAM1, were incubated for 30 min at 37°C and pH 7.2 before the addition of 101.6 μmol/L Ala-AMC (~1 Kₐ; table 1). The remaining experimental conditions were equal to those above described. Control assays were prepared by pre-incubating the enzyme with the same volume of the solvent used to dissolve each inhibitor. Residual activity was defined as the ratio between the reaction rate in the presence of the inhibitor and the rate of the control.

Statistical analyses

Normal distribution and homogeneity of variances for the data were verified using the Statistica software (version 8.0; StatSoft Inc. [http://www.statsoft.com]) with default parameters. The same software was used for means comparison by the Dunnett test.
RESULTADOS

We determined the main kinetic characteristics of rPfAM1, previously obtained by our group (González-Bacerio et al., 2014), by using aminoacyl-AMC fluorogenic substrates. This is essential to further correlate the results of inhibition of the recombinant enzyme with the inhibition of nPfAM1 in the context of intact live parasites (this last assay requires a fluorogenic substrate). Since some unknown amount of Zn$^{2+}$ may be lost from the active site during purification of rPfAM1 by IMAC (González-Bacerio et al., 2014), we also assessed the effect of Zn$^{2+}$ concentration on the EA of the purified enzyme. No augment of activity was registered in the range 10 nmol/L - 1 μmol/L ZnCl$_2$ (fig. 1), but inhibition at concentrations higher than 1 μmol/L was obtained, indicating that addition of Zn$^{2+}$ to the enzymatic preparation is unnecessary for the activity assays. As expected, the optimum pH for the AP activity of rPfAM1 toward Ala-, Leu-, Arg- and Met-AMC is in the 7.2-7.4 range (fig. 2).

Determination of the kinetic parameters of rPfAM1 showed that the highest $k_{cat}$ values at both pH were obtained with Ala at the P1 position of the aminoacyl-AMC substrates (table 1). At pH 7.2, the $k_{cat}$ against Met-, Arg- and Leu-AMC were 2.7, 2.1 and 3.4 times lower, respectively, than the $k_{cat}$ for Ala-AMC. However, at pH 5.2 the values were 8, 4 and 16 times lower, respectively. In contrast, the lowest $K_M$ values at both pH were obtained with Met-AMC. Comparing with Met-AMC, the $K_M$ values were 1.6, 4.6 and 2.6 times higher, at pH 7.2, and 2, 48 and 1.3 times higher, at pH 5.2, for Arg-, Ala- and Leu-AMC, respectively (table 1).

The $K_M$ values increased only 1.5-1.9 times at pH 5.2 compared to those at pH 7.2 ($K_M$ for Leu-AMC even decreases slightly), with the exception of Ala-AMC, for which a 16-fold augment was observed. In contrast, catalytic efficiencies were more affected due to the pH fall, with diminutions of 16-40 times in $k_{cat}$ except for Ala-AMC, with a 8-fold decrease. In accordance with these variations, the specificity constants ($k_{cat}/K_M$) diminished 31-137 times at acid pH. As is shown from the $k_{cat}/K_M$ values, the substrate specificity of PfAM1 at pH 7.2 for the four P1 amino acids tested was: Met > Arg > Ala > Leu, which suffered a slight variation at pH 5.2: (Met = Arg) > Leu > Ala (table 1). On the other hand, rPfAM1 was not sensitive to PMSF, E64 and pepstatin A, but it was inhibited by amastatin, 1,10-phenanthroline and bestatin (fig. 3).

**Figure 1.** Effect of Zn$^{2+}$ concentration on aminopeptidase activity of rPfAM1. Assays were performed with 34.7 μmol/L Leu-AMC at pH 7.2. Activity in the absence of exogenous Zn$^{2+}$ was considered as 100%. Values are represented as means (n = 3). Asterisks represent significant differences by the Dunnett test (p < 0.05) with activity in the absence of Zn$^{2+}$.

**Figure 2.** Effect of pH on aminopeptidase activity of rPfAM1. Assays were performed with 406.3 μmol/L Ala-AMC (circles), 347.1 μmol/L Leu-AMC (squares), 302 μmol/L Arg-AMC (diamonds) and 326.7 μmol/L Met-AMC (triangles). Activity was normalized using the maximum activity level (corresponding to pH = 7.2-7.4) as 100%. Values are represented as means (n = 3).
Moreover, the near neutral pH optimum here shown for the AP activity of a metallo-AP from the larvae of the silkworm Bombyx mori is inhibited at more than 100 μmol/L Zn²⁺ (Hua et al., 1998). Moreover, the near neutral pH optimum here shown for the AP activity of rPFAM1 (figure 2) was also previously observed when using the chromogenic substrate Leu-aminoacyl-p-nitroanilide (pNA) (González-Bacero et al., 2014), and is in agreement with previous reports for nPFAM1 and rPFAM1.

DISCUSSION

The kinetic characteristics determined in this work for rPFAM1 are crucial in order to confirm that this enzyme is similar to nPFAM1, in terms of their kinetic behaviour towards aminoacyl-AMC conjugates. Such condition is necessary to identify potential inhibitors of nPFAM1 using rPFAM1 as a target.

First, we detected that rPFAM1 is inhibited by Zn²⁺ at concentrations > 1 μmol/L (figure 1), which is consistent with our previous characterization of this enzyme using chromogenic substrates (González-Bacero et al., 2014). The inhibition of metallo-APs at high Zn²⁺ concentrations has been reported by various authors. For example, a neutral AP from the larvae of the silkworm Bombyx mori is inhibited at more than 100 μmol/L Zn²⁺ (Hua et al., 1998). Moreover, the near neutral pH optimum here shown for the AP activity of rPFAM1 (figure 2) was also previously observed when using the chromogenic substrate Leu-aminoacyl-p-nitroanilide (pNA) (González-Bacero et al., 2014), and is in agreement with previous reports for nPFAM1 and rPFAM1.
the recombinant variant obtained in 2009: rPFAM1\(^{195-1085}\) (Allary et al., 2002; McGowan et al., 2009). These results are compatible with the cytosolic localization previously proposed for the endogenous AP in *P. falciparum* (Kolakovich et al., 1997; Gavigan et al., 2001; Allary et al., 2002; Azimzadeh et al., 2010).

The K\(_M\) values obtained in this work toward Arg-, Leu- and Ala-AMC substrates, at near-neutral pH, are closer to those reported by Allary et al. (2002) for rPFAM1 (100, 60 and 200 \(\mu\)mol/L, respectively) than those obtained with rPFAM1\(^{195-1085}\) (717, 330 and 889 \(\mu\)mol/L, respectively; McGowan et al., 2009) (table 1). In this sense, our recombinant variant of PFAM1 is similar to rPFAM1. On the other hand, neither Met-AMC nor acidic conditions in the kinetic assays were tested in those previous works, being this report the first one about the kinetic parameters of PFAM1 at acid pH toward aminoacyl-AMC substrates. Similarly, k\(_{cat}\) values have not been reported for the native enzyme toward these substrates. Hence, here we performed a deeper kinetic characterization of the enzyme.

The k\(_{cat}\) values here obtained at pH 7.2 are 53-134 times higher than those reported for rPFAM1\(^{195-1085}\) (McGowan et al., 2009) (table 1). The discrepancies in K\(_M\) and k\(_{cat}\) between the recombinant variant of PFAM1 obtained by our group (González-Bacerio et al., 2014) and the form produced by McGowan et al. (2009): rPFAM1\(^{195-1085}\), may be a consequence of assessing the kinetic parameters of the latter at pH 7.5, which is different to the pH optimum reported for this enzyme (pH 7.0; McGowan et al., 2009). The disparities in k\(_{cat}\) may be also caused by dissimilar percentages of active molecules in both enzyme preparations (we assumed 100 % activity to calculate k\(_{cat}\)). Since a possible cause of overestimating the k\(_{cat}\) is the underestimation of the enzyme concentration, we assessed the rPFAM1 concentration by two distinct methods (Bradford method and absorbance at 280 nm), which allowed the accurate quantification of the protein.

Since the effect of pH upon the rPFAM1 kinetic parameters is common for substrates with basic (arginine) or non-ionizable (methionine, leucine) P1 side chains (table 1), we reject the pH-dependent variation of electrostatic interactions between the enzyme active site and the P1 side chain as the cause of the catalytic efficiency impairment at pH 5.2. Our data could be influenced by a conformational change, and/or an unfavourable protonation state of catalytic residues, in the rPFAM1 active site at acid pH, resulting in a general negative effect on catalysis and a substantial loss of affinity for P1 residues with small side chains, such as alanine. Since the specificity constants remain at high values at pH 5.2 (>10\(^7\) Lmol\(^{-1}\)s\(^{-1}\); table 1), our kinetic results do not allow discarding a possible localization of nPFAM1 in the parasite acidic food vacuole, as was previously suggested (Dalal and Klemba, 2007; Harbut et al., 2011; Ragheb et al., 2011). In this sense, high K\(_M\) values at acid pH toward peptides containing alanine at P1 position (table 1) would be compensated by high concentrations of such peptide substrates in the *P. falciparum* digestive vacuole (alanine constitutes 12.46 % amino acids in human hemoglobin; McGowan et al., 2009), as was previously reasoned (Ragheb et al., 2011).

The obtainment of the highest k\(_{cat}\) values with the Ala-AMC substrate (table 1) is consistent with the alternative denomination of this enzyme as alanyl AP (Skinner-Adams et al., 2010). Variation of the rPFAM1 kinetic parameters at near-neutral pH in function of the P1-residue identity (table 1) is similar to that observed with rPFAM1\(^{192-1085}\) toward aminoacyl-Ala natural dipeptide substrates (Dalal et al., 2012). In the present work, the kinetic parameters varied as follows (in descending order): K\(_M\): Ala > Leu > Arg > Met; k\(_{cat}\): Ala > Arg > Met > Leu; and k\(_{cat}\)/K\(_M\): Met > Arg > Ala > Leu. In the reference study, the order is the same for K\(_M\), whereas slightly different for k\(_{cat}\): Ala > (Arg = Met = Leu) and k\(_{cat}\)/K\(_M\): Met > Arg > Leu > Ala (Dalal et al., 2012). These coincidences confirm the relevance of the P1 site for the PFAM1 substrate specificity (Allary et al., 2002; McGowan et al., 2009; Dalal et al., 2012; Poreba et al., 2012; González-Bacerio et al., 2014) and endorses using these aminoacyl-AMC substrates to assess the inhibition of the native and recombinant enzymes. The substrate specificity exhibited by rPFAM1 against P1 natural amino acids (table 1) also matches that of ETAPN1, the PFAM1 ortholog from the avian apicomplexan parasite *Eimeria tenella* (Gras et al., 2014).

The inhibition profile of rPFAM1 (fig. 3) is similar to that of nPFAM1 and typical of neutral and basic metallo-APs (Allary et al., 2002). All results derived from the kinetic characterization of rPFAM1 using aminoacyl-AMC fluorogenic substrates, like those previously obtained with aminoacyl-pNA chromogenic substrates (González-Bacerio et al., 2014), indicate that our recombinant protein has a kinetic behavior which is very similar to that of the native enzyme. Consequently,
rPfAM1 can be used as a model of nPfAM1 to search for inhibitors. In addition, the kinetic parameters obtained here with the fluorogenic peptides, at near-neutral and acid pH (the latter for the first time), allow the use of these substrates for the evaluation ofnPfAM1 activity and its inhibition directly in parasites.

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CITED LITERATURE


